Exhibit B

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Proteolysis of Factor Va by Factor Xa and Activated Protein C*

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Bovine Factor Va, produced by selective proteolytic cleavage of Factor V by thrombin, consists of a heavy chain (D chain) of M_r = 94,000 and a light chain (E chain) of M_r = 74,000. These peptides are noncovalently associated in the presence of divalent metal ion(s). Each chain is susceptible to proteolysis by activated protein C and by Factor Xa. Sodium dodecyl sulfate electrophoretic analysis indicates that cleavage of the E chain by either activated protein C or Factor Xa yields two major fragments: M_r = 30,000 and M_r = 48,000. Amino acid sequence analysis indicates that the M_r = 30,000 fragments have identical NH_2-terminal sequences and that this sequence corresponds to that of intact E chain. The M_r = 48,000 fragments also have identical NH_2-terminal sequences, indicating that activated protein C and Factor Xa cleave the E chain at the same position. Sodium dodecyl sulfate electrophoretic analysis indicates that activated protein C cleavage of the D chain yields two products: M_r = 70,000 and M_r = 24,000. Amino acid sequence analysis indicates that the M_r = 70,000 fragment has the same NH_2-terminal sequence as intact D chain, whereas the M_r = 24,000 fragment does not. Factor Xa cleavage of the D chain also yields two products: M_r = 56,000 and M_r = 45,000. The M_r = 56,000 fragment corresponds to the NH_2-terminal end of the D chain and Factor V. Functional studies have shown that both chains of Factor Va may be entirely cleaved to products by Factor Xa without loss of activity, whereas activated protein C cleavage results in loss of activity. Since activated protein C and Factor Xa cleave the E chain at the same position, the cleavage of the D chain by activated protein C is responsible for the inactivation of Factor Va.

Coagulation Factor Va is an essential nonenzymatic component of prothrombinase, the catalyst responsible for the effective activation of prothrombin to thrombin in the clotting cascade (1, 2). Consisting of the serine protease Factor Xa and the nonenzymatic cofactor Factor Va associated with phospholipid in the presence of Ca^{2+}, the complete prothrombinase complex is able to catalyze the conversion of prothrombin to thrombin at a rate 10^5-fold greater than that of Factor Xa alone (3).

Regulation of the cofactor activity of Factor Va is achieved through proteolysis. Both the procofactor Factor V and the active cofactor Factor Va are susceptible to proteolytic cleavages catalyzed by coagulation proteases.

Single chain Factor V (M_r = 330,000) is activated by limited and specific proteolysis. Both thrombin (1-3) and Factor Xa (4) can serve as activators, although each catalyzes a different set of bond cleavages and produces different active species (4). The Factor Va resulting from activation by thrombin has been more extensively studied (9). It consists of two polypeptide chains, one of M_r = 94,000 and one of M_r = 74,000. Although thrombin activates Factor V at a rate at least 100-fold greater than does Factor Xa, Factor Xa likely serves as the primary activator of Factor V during initial stages of thrombin production when little or no thrombin is present (4).

Factor Va is inactivated by limited proteolysis which is catalyzed by activated protein C (5-7, 10). Evidence indicates that the inactivation of Factor Va is associated with cleavage of its heavy chain, whereas the light chain is cleaved at a slower rate (5-8).

Both chains of Factor Va are also proteolytically processed by Factor Xa, and cleavage of the light chain does not have a negative effect on the activity of the cofactor (8). When subjected to SDS[1]-polyacrylamide slab gel electrophoresis, the two fragments (one of M_r = 30,000 and one of M_r = 48,000) from cleavage of the light chain of Factor Va by Factor Xa appear in positions apparently the same as those for the two fragments from cleavage of the light chain by activated protein C. Cleavage of the heavy chain of Factor Va by Factor Xa yields fragments (one of M_r = 56,000 and one of M_r = 45,000) clearly different from those (one of M_r = 70,000 and one of M_r = 24,000) produced by activated protein C cleavage of the heavy chain (8).

Factor VIIIa is another cofactor of the clotting cascade whose activity is regulated by proteolysis. Factors VIIIa and Va have many similar structural and functional characteristics. Both are produced from a large procofactor through cleavage by thrombin, both are about the same size and consist of a heavy chain and a light chain, both contribute greatly to the activity of a proteolytic complex while not exhibiting proteolytic activity on their own, and both are inactivated by activated protein C. Underlying this commonality is an apparent homology of primary structure (15, 19).

We have determined amino-terminal sequences of the fragments of activated protein C and Factor Xa cleavages of the components of Factor Va, and we have made observations concerning the effects of these cleavages on Factor Va activity. Together with earlier evidence, these new data allow us to deduce the relative positions from which the various frag-

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[1] The abbreviations used are: SDS, sodium dodecyl sulfate; MES, 4-morpholineethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography; DAPA, 5-dimethylaminonaphthalene-1-sulfonylarginine-*N*-(3-ethyl-1,5-pentanedylamine.

ments originated and to confirm which cleavage results in the inactivation of Factor Va. In addition, we have identified further sequence homology between Factors V and VIII.

EXPERIMENTAL PROCEDURES

Isolations of Proteins—Bovine prothrombin, Factor X, and protein C were isolated from the dissolved precipitate of barium citrate-adsorbed plasma by initial resolution on a column of QAE-Sephadex (5, 14). Prothrombin and Factor X were further purified by chromatography on DEAE-cellulose (20) as described (14). Protein C was further purified by chromatography on heparin-Sepharose (5), followed by chromatography on dextran sulfate-agarose (21) as described (13). Prothrombin was activated by incubation with prothrombinase assembled from purified components. Thrombin was then isolated by chromatography on a column of SP-Sephadex C-50 as described (22). Factor X was activated using purified Factor X activator from Russell's viper venom (23), and Factor Xa was isolated as described (14). Protein C was activated by incubation with α-thrombin and purified as described (24).

Bovine Factor V was isolated as described (25). Factor V was activated by α-thrombin as described (13). Factor Va and its individual components D (heavy chain) and E (light chain) were isolated by any one of three methods: ion exchange on QAE-Sephadex as described by Esmon (1), the method of Krishnaswamy *et al.* (13) (as described, this method applies only to the individual components D and E but works for intact Factor Va by replacing the EDTA by 2 mM $CaCl_2$), or a third method which is a modification of the immunoadsorption step for the isolation of human Factor V (25). In this last case, it was discovered that whereas the anti-human Factor V monoclonal antibody does not bind bovine Factor V, it does bind bovine Factor Va. Factor V was activated in 0.02 M Tris/HCl, 0.15 M NaCl, pH 7.4, by treatment with 10 NIH units/ml α-thrombin at 37 °C for 10 min. $CaCl_2$ was then added to 2 mM, and the thrombin was inactivated by addition of diisopropyl fluorophosphate to 10 mM. The solution was applied to the immunoadsorbent column equilibrated in Tris/saline/$CaCl_2$. After the initial flow-through peak and buffer wash, Factor Va was eluted from the column with buffer made 1.5 M in NaCl. The procedure for isolation of components D and E was identical up through washing the column. At that point, the buffer was changed to contain 10 mM EDTA instead of $CaCl_2$. One column volume of this buffer was allowed to enter the column, and flow was stopped for 10 min and then continued. Component D was eluted with this EDTA buffer. After the component D peak was eluted, the column was washed with buffer made 1.5 M NaCl to elute component E. When Factor V was activated, component E appeared most often as a doublet, both in the initial set of fragments (Fig. 1) and when isolated as part of Factor Va or by itself. The two species may be separated on a Mono-S column (Pharmacia P-L Biochemicals fast protein liquid chromatography system). A solution of component E at 0.2 mg/ml was dialyzed against 20 mM Tris/MES, 5 mM EDTA, pH 6.0, and applied at 0.5 ml/min to the Mono-S (20:10) column equilibrated in the same buffer. The column was washed with 5 ml of buffer, and then a linear gradient from 0 to 0.6 M NaCl in Tris/MES was run in 30 min, followed by a gradient of 0.6–1.0 M NaCl in Tris/MES in 5 min. A peak eluting at 0.45 M NaCl contained the species of lower molecular weight, whereas the species of higher molecular weight eluted at slightly greater than 0.6 M NaCl. Concentrations of Factor Va and components D and E by $(NH_4)_2SO_4$ precipitation and storage in 50% glycerol were as described (13). The molecular weights and extinction coefficients ($E^{0.1\%}_{280\ nm}$) for the proteins used in this study were, respectively: Factor V, 330,000 and 0.96 (26); component D, 94,000 and 1.22 (27); and component E, 74,000 and 1.22 (27).

Isolation of Proteolytic Fragments of Components D and E—Proteolytic digestion of components D and E by Factor Xa or activated protein C was performed in Tris/saline at 37 °C at substrate:protease molar ratios of approximately 10:1. Appropriate digestion times were determined by screening time courses and running gels. An example is shown in Fig. 2 where component D at 2.6 μM was incubated with activated protein C at 0.3 μM in Tris/saline at 37 °C. Aliquots (6 μg) were removed at specific times, added to equal volumes of glacial acetic acid, diluted 10-fold with water, dried in a Savant Speed Vac apparatus, and prepared for SDS-PAGE. Fragments of components D and E were isolated either by HPLC or by electroelution. Reverse-phase HPLC was successful for isolation of fragments of component E after Factor Xa or activated protein C cleavage. An Altex C3 column was used with a linear gradient of 20–40% solvent B in 10 min at 0.7 ml/min (solvent A = 0.5% trifluoroacetic acid in H_2O; solvent B = 0.5% trifluoroacetic acid in CH_3CN). This method separated the fragment of M_r = 30,000 from the doublet of M_r = 48,000/46,000 but did not resolve the members of the doublet. For fragment of component D, reverse-phase HPLC was not successful, and electroelution of proteins from polyacrylamide gels was performed as described by Hunkapillar *et al.* (28).

Sequence Analysis—Edman degradation was performed on an Applied Biosystems gas-phase sequenator by procedures similar to those described by Hewick *et al.* (29). Phenylthiohydantoin analysis was performed by HPLC. The amount of sample applied to the sequenator was estimated by A_{280} using $E^{1\%}_{280\ nm}$ = 1.0 when enough material was present. In other cases, it was estimated by Coomassie Blue staining intensity in a gel using the parent component as a standard.

Assay of Factor Va Activity—Factor Va activity was assayed by continuously measuring the conversion of prothrombin to thrombin in the presence of DAPA (3, 4). Excitation was at 280 nm, and reactions were initiated by adding Factor Va.

Gel Electrophoresis—Analytical slab gel PAGE was performed in 5–15% gradient gels using the method of Laemmli (30). Gels were stained either with Coomassie Brilliant Blue R-250 or by a modification of the silver-staining procedure of Merrill *et al.* (31) as described (9). PAGE for electroelution was performed in essentially the same way, but with sample preparation modifications as suggested by Hunkapillar *et al.* (28).

RESULTS

Proteolytic Processing of Factor V and Va Components—When bovine Factor V is activated by thrombin, component E (the carboxyl terminus-derived light chain) most often appears in SDS-polyacrylamide gels as a doublet with one member of M_r = 74,000 and one of M_r = 72,000 (Fig. 1, *lane 3*). The relative amounts of the two members of the doublet vary from preparation to preparation. Both species of com-

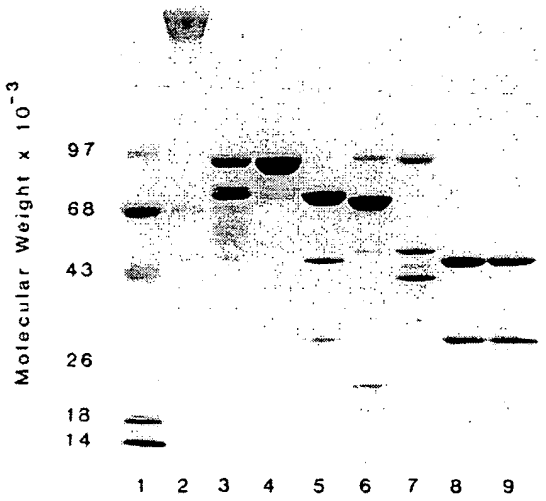

FIG. 1. **Products of proteolysis of Factor V and Va components.** Factor V and components D and E of Factor Va were treated with protease as described below. All reactions took place at 37 °C in 0.02 M Tris/HCl, 0.15 M NaCl, pH 7.4 SDS-polyacrylamide slab gel electrophoresis was performed on the reduced samples as described under "Experimental Procedures." Conditions were: Coomassie Blue stain. *Lane 1*, molecular weight standards, 2 μg each; *lane 2*, bovine Factor V, 15 μg; *lane 3*, sample as in *lane 2* but after 10 min of incubation with thrombin at a concentration of 10 NIH units/ml; *lane 4*, component D isolated by immunoadsorption as described under "Experimental Procedures," 15 μg; *lane 5*, component E singlet isolated by immunoadsorption followed by Mono-S ion-exchange chromatography as described under "Experimental Procedures," 15 μg; *lane 6*, sample as in *lane 4* (component D) but after 20 min of incubation with 1 μg of activated protein C; *lane 7*, sample as in *lane 4* (component D) but after 3 h of incubation with 1 μg of Factor Xa; *lane 8*, sample as in *lane 5* (component E) but after 3 h of incubation with 1 μg of activated protein C; *lane 9*, sample as in *lane 5* (component E) but after 3 h of incubation with 1 μg of Factor Xa.

ponent E are isolated by any one of the three methods described under "Experimental Procedures." The component E doublet is usually accompanied by a set of fragments: a doublet at M_r = 48,000 and 46,000 and a singlet at M_r = 30,000. These fragments are faintly visible in *lane 3* of Fig. 1, and the isolated M_r = 48,000/46,000 doublet is shown in *lane 8* of Fig. 3. The members of the component E doublet can be resolved by ion-exchange chromatography on a Pharmacia P-L Biochemicals Mono-S column as described under "Experimental Procedures." In the process, the doublet at M_r = 48,000/46,000 is also separated, with the fragment of M_r = 46,000 eluting in the same peak as the polypeptide of M_r = 72,000 and the fragment of M_r = 48,000 eluting with the polypeptide of M_r = 74,000. A fragment of M_r = 30,000 elutes with each pair. An example of the M_r = 72,000/46,000/30,000 set is shown in *lane 5* of Fig. 1.

When the component E doublet is subjected to proteolysis by either Factor Xa or activated protein C, the doublet disappears with a corresponding increase in the fragment doublet at M_r = 48,000/46,000 and the fragment singlet at M_r = 30,000 (not shown). If an isolated member of the component E doublet is similarly treated with Factor Xa or activated protein C, the corresponding singlet at M_r = 48,000 or 46,000 appears along with the fragment of M_r = 30,000. An example of this is seen in *lane 8* and *9* of Fig. 1, which show the results of allowing the M_r = 72,000 member of the component E doublet to be cleaved by activated protein C or Factor Xa, respectively. Some residual parent molecule at M_r = 72,000 has been left for reference, although it is possible to completely eliminate it. For both activated protein C and Factor Xa proteolysis, the major products at M_r = 46,000 and 30,000 migrate the same in SDS-polyacrylamide gels.

On SDS-polyacrylamide gels, component D (the amino terminus-derived heavy chain) of Factor Va appears primarily as a single band (Fig. 1, *lane 4*), and the fragments from its proteolysis by activated protein C or Factor Xa are different (Fig. 1, *lanes 6* and *7*). Fig. 2 shows a time course of proteolysis of component D by activated protein C. The parent molecule is rapidly cleaved to fragments of M_r = 70,000 and 24,000, with the fragment of M_r = 24,000 disappearing as one of M_r = 20,000 appears. A typical product set is shown in *lane 6* of Fig. 1 with some residual parent molecule for reference.

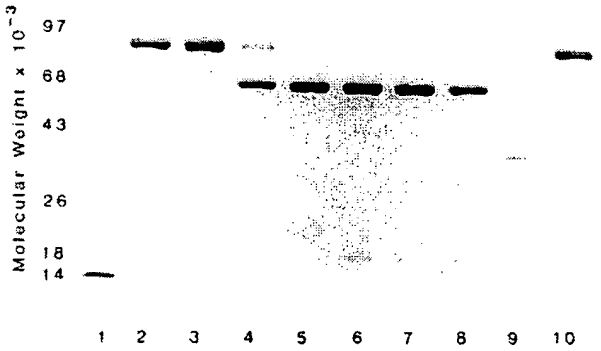

FIG. 2. **Time course of component D proteolysis by activated protein C.** Component D (2.6 μM) was incubated with activated protein C (0.3 μM) at 37 °C in 0.02 M Tris/HCl, 0.15 M NaCl, pH 7.4. At various times, an aliquot was removed and prepared (nonreduced) for SDS-polyacrylamide gel electrophoresis as described under "Experimental Procedures." Conditions were: 6 μg/lane; Coomassie Blue stain. *Lane 1*, molecular weight standards, 2 μg each; *lane 2*, component D, no activated protein C; *lanes 3–9*, component D plus activated protein C at 0 s, 10 s, 10 min, 20 min, 1 h, 2 h, 4 h, and 20 h; *lane 10*, component D, no activated protein C at 20 h.

The Factor Xa-catalyzed proteolysis of component D occurs more slowly. As shown in *lane 7* of Fig. 1, the major products of Factor Xa cleavage of component D are fragments of M_r = 56,000 and 45,000.

Isolation of Polypeptide Fragments for Sequencing—The major fragments observed for Factor Xa or activated protein C proteolysis of components D and E were isolated by reverse-phase HPLC or electroelution. When the component E doublet was cleaved by either activated protein C or Factor Xa, reverse-phase HPLC successfully resolved the fragment of M_r = 30,000 from the fragment doublet of M_r = 48,000 and 46,000, but the members of the fragment doublet were not resolved. The products of such an isolation are shown in *lanes 7–9* of Fig. 3. In some cases, individual members of the fragment doublet were isolated by electroelution, shown in *lane 6* of Fig. 3.

The fragments of proteolysis of component D by either activated protein C or Factor Xa were not resolved by reverse-phase HPLC using various columns and solvent systems. The major fragments were isolated by electroelution and are shown in *lanes 2–5* of Fig. 3. The fragment of M_r = 20,000 from activated protein C cleavage of component D was also isolated by electroelution (not shown).

Amino-terminal Sequences of Proteolytic Fragments—The results of Edman degradation and phenylthiohydantoin analysis are shown in Table I.

The fragments of M_r = 30,000 from the cleavage of component E by either activated protein C or Factor Xa yielded the same sequence, and this sequence was identical to that of the intact component E (15). The fragment doublet from Factor Xa cleavage of component E (Fig. 3 *lane 8*) yielded a single sequence. The electroeluted fragment of M_r = 48,000 from activated protein C cleavage of component E yielded a sequence from positions 2–12 which was identical to that found in the doublet. An electroeluted single fragment of M_r = 46,000 from activated protein C cleavage of component E had an identical sequence in positions 2–8. It appears then

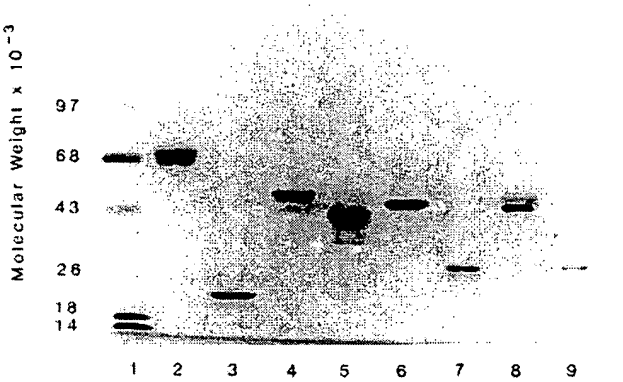

FIG. 3. **Isolated fragments used for sequencing.** Sample preparations and electrophoresis were as described in the legend to Fig. 1. Sample loads were 2–6 μg/lane. *Lane 1*, molecular weight standards; *lane 2*, fragment of M_r = 70,000 from activated protein C cleavage of component D; *lane 3*, fragment of M_r = 24,000 from activated protein C cleavage of component D; *lane 4*, fragment of M_r = 56,000 from Factor Xa cleavage of component D; *lane 5*, fragment of M_r = 45,000 from Factor Xa cleavage of component D; *lane 6*, fragment of M_r = 48,000 from activated protein C cleavage of component E; *lane 7*, fragment of M_r = 30,000 from activated protein C cleavage of component E; *lane 8*, fragment doublet of M_r = 48,000/46,000 from Factor Xa cleavage of component E; *lane 9*, fragment of M_r = 30,000 from Factor Xa cleavage of component E. Polypeptides in *lanes 2–6* were isolated by electroelution. Those in *lanes 7–9* were isolated by reverse-phase HPLC.

TABLE I

Amino-terminal sequences of proteolytic fragments of components D and E

Cycle	Component D					Intact D[b]	Intact E[b]	Component E			
	APC[a]			Factor Xa				APC		Factor Xa	
	70[c]	24	20	56	45			30	48	30	48
	(NQ)[d]	(500)	(80)	(NQ)	(NQ)			(NQ)	(120)	(10,000)	(6,100)
1	A (125)	—	—	—	—	A	S	—	—	S (NQ)	—
2	K (59)	I (125)	I (44)	K (260)	—	K	N	—	A (48)	N (1,700)	A (855)
3	L (82)	—	—	L (102)	—	L	T	T (NQ)	S (NQ)	T (NQ)	S (NQ)
4	R (23)	R (94)	R (16)	—	L (54)	R	G	G (165)	S (NQ)	G (2,100)	S (NQ)
5	Q (91)	A (185)	A (25)	Q (NQ)	D (94)	Q	N	N (100)	E (NQ)	N (1,200)	E (550)
6	F (56)	A (185)	A (25)	F (71)	N (120)	F	R	R (124)	V (NQ)	R (1,400)	V (554)
7	Y (53)	D (100)	D (13)	Y (61)	F (100)	Y	K	K (141)	K (NQ)	K (NQ)	K (634)
8	V (58)	I (155)	I (22)	V (70)	S (NQ)	V	Y	Y (176)	N (NQ)	Y (3,000)	N (202)
9	A (67)	E (75)	E (11)	A (60)	N (114)	A	Y	Y (154)	S (NQ)	Y (3,200)	S (NQ)
10	A (70)	Q (114)	Q (NQ)		—	A	Y	Y (172)	—	Y (3,200)	H (333)
11	Q (90)	Q (114)			I (108)	Q	I	I (113)	E (NQ)	I (3,300)	E (370)
12	S (NQ)	A (108)			G (41)	S	A	A (116)	F (NQ)	A (2,600)	F (240)
13	I (20)	V (132)			K (130)	I	A	A (123)		A (2,700)	H/A (208/120)
14	R (10)	F (118)			—	R	E	E (NQ)		E (3,200)	A (166)
15	W (7)	A (100)			Y (58)	W	E	E (NQ)		E (3,200)	I/P (102/60)
16	N (19)	V (98)			K (100)	N	I	I (98)		I (2,600)	N (242)
17	Y (8)	F (102)			K (130)	Y	S	S (NQ)		S (NQ)	G (252)
18		D (38)			V (NQ)	R	W	W (16)			M (265)
19		E (52)				—	D				I (375)
20		N (28)				P	Y				Y (350)
21		K (48)				E	S				N (180)
22		R (NQ)				S	K				L (203)
23		W (NQ)				T	F				P (213)
24		Y (20)				H	V				G (204)
25		I (15)				L	Q				L (273)
26		E (15)									R (56)
27		D (5)									M (258)

[a] APC, activated protein C; NQ, not quantitated; —, not identified.
[b] From Ref. 19.
[c] M_r = 70,000 fragment, etc.
[d] The numbers in parentheses indicated picomoles at the given cycle.

that both members of the doublet have the same amino-terminal sequence, and the amino-terminal sequences of both the activated protein C and Factor Xa cleavage products are identical.

For component D, the fragments of M_r = 70,000 (from activated protein C cleavage) and 56,000 (from Factor Xa cleavage) are derived from the amino-terminal portion of the parent molecule as indicated by their sequence identity with the intact component D. The fragments of M_r = 24,000 and 20,000 from activated protein C cleavage of component D have amino-terminal sequences which are identical to each other, indicating that the fragment of M_r = 20,000 is derived from the fragment of M_r = 24,000 by an activated protein C cleavage quite near the carboxyl terminus. The amino-terminal portion of the parent molecule as indicated by their sequence of the fragment of M_r = 45,000 is different from all other sequences in the set, consistent with its being the result of a unique cleavage by Factor Xa near the middle of component D.

Sequence Homology of Bovine Factor V Fragments with Human Factor VIII—Because it had already been demonstrated that bovine Factor V and porcine Factor VIII contain segments of significant sequence homology (15, 19), the sequence data of Table I were compared to the amino acid sequence derived from the nucleic acid sequence of human Factor VIII (16, 17). The results of the search are shown in Table II. There are clearly segments of sequence homology between bovine Factor V and human Factor VIII at positions in the Factor VIII molecule that correspond reasonably well to the positions in the Factor V molecule at which cleavages occur.

Relationship of Proteolysis to Inactivation of Factor Va—Because of the interest in the relationship between proteolysis and control of Factors V and Va function, the functional effects of Factor Xa cleavage of Factor Va were explored.

Factor Va (0.3 μM) was incubated at 37 °C with either activated protein C (0.3 μM) or Factor Xa (0.3 μM) in 0.02 M Tris/HCl, 0.15 M NaCl, 2 mM $CaCl_2$, 43 μM DAPA, pH 7.4. At various times, an aliquot (10 μl) was added to a cuvette containing 2.0 ml of a solution of 0.02 M Tris/HCl, 0.15 M NaCl, 2 mM $CaCl_2$, 3 μM DAPA, 1.39 μM prothrombin, and 15 nM Factor Xa. The final Factor Va concentration was 1.5 nM. The increase of fluorescence intensity was measured as a function of time. At the same time that aliquots were taken for functional assays, aliquots were prepared for SDS-polyacrylamide gel electrophoresis. The small amount of protein present made a silver-staining technique necessary. The results of the experiment are shown in Fig. 4.

Lanes 1 and *6* of Fig. 4 (*lower*) and the corresponding curves of Fig. 4 (*upper*) indicate that in the absence of protease, Factor Va activity was stable during the course of the experiment and that no chain degradation took place. In the presence of Factor Xa (*lanes 2, 4,* and *7* and the corresponding curves), first component E and then component D are completely cleaved without any loss of cofactor activity. In parallel incubations with activated protein C (*lanes 3, 5,* and *8* and the corresponding curves), both inactivation and chain degradation take place. It should be noted, however, that even when both components of Factor Va are completely cleaved by activated protein C, there is residual Factor Va activity of about 30% of that present at the beginning of the experiment.

TABLE II

Sequence homology between bovine Factor V, human Factor VIII, and human cerruloplasmin

The data and numbering for human Factor VIII (HFVIII) were from Ref. 17, and those for cerruloplasmin were from Ref. 18. The asterisks indicate that the amino acid at that position was not identifiable, and the dashes indicate that a gap was introduced.

HFVIII (beginning at position 412)	L N N G P Q R I G R K Y K K
Fragment of M_r = 45,000	L D N F S N * I G K * Y K K
Human cerruloplasmin	- - Q G T T R I G G S Y K K
HFVIII (beginning at position 576)	F - S V F D E N R S W Y L T E
Fragment of M_r = 24,000	F - A V F D E N K R W Y I E D
Human cerruloplasmin	F P T V F D E N E S L L L E D
HFVIII (beginning at position 1918)	F H A I N G Y I M D T L P G L V M
Fragment of M_r = 48,000	F H A I N G M I Y - N L P G L R M
Human cerruloplasmin	M H A I N G R M F G N L Q G L T M

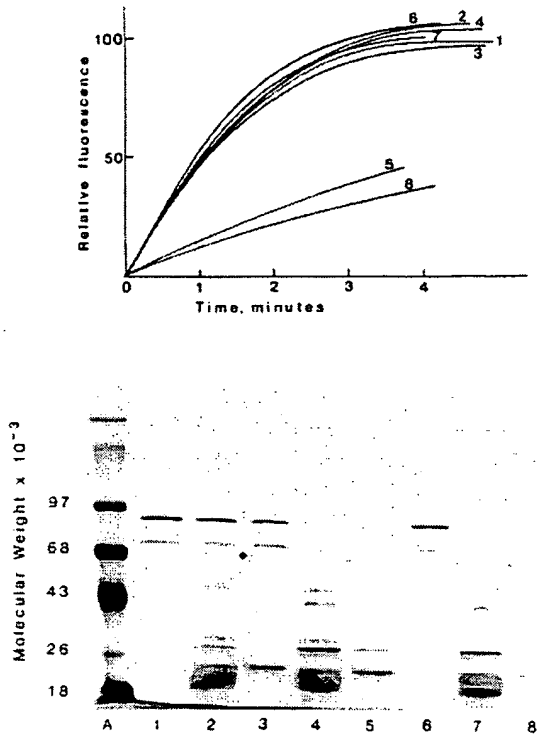

FIG. 4. **Cofactor activity of Factor Va after cleavage by Factor Xa or activated protein C.** *Upper*, assay of cofactor activity using DAPA. Factor Va (0.3 μM) was incubated at 37 °C in 0.02 M Tris/HCl, 0.15 M NaCl, pH 7.4, containing DAPA (43 μM), $CaCl_2$ (2 mM), and either Factor Xa (0.3 μM) or activated protein C (0.3 μM). At various times, an aliquot (10 μl) was added to a cuvette containing 2.0 ml of 0.02 M Tris/HCl, 0.15 M NaCl, pH 7.4, containing prothrombin (1.39 μM), DAPA (3.0 μM), PCPS (75% phosphatidyl choline, 25% phosphatidyl serine vesicles (12 μM), $CaCl_2$ (2 mM), and Factor Xa (15 nM). The final Factor Va concentration was 1.5 nM). The increase in fluorescence intensity was measured as a function of time. *Curve 1*, Factor Va only, before addition of protease and before incubation at 37 °C; *curve 2*, Factor Va + Factor Xa immediately upon adding Factor Xa; *curve 3*, Factor Va + activated protein C upon adding activated protein C; *curve 4*, Factor Va + Factor Xa at 2 h; *curve 5*, Factor Va + activated protein C at 2 h; *curve 6*, Factor Va after 4 h of incubation, no protease; *curve 7*, Factor Va + Factor Xa at 4 h; *curve 8*, Factor Va + activated protein C at 4 h. *Lower*, SDS-polyacrylamide gel electrophoresis as described in the legend to Fig. 1 of 60-μl aliquots of Factor Va during incubations with Factor Xa or activated protein C. Conditions were: reduced samples; silver stained. *Lane A*, molecular weight standards; *lanes 1–8* correspond to *curves 1–8* above.

DISCUSSION

The proteolysis of Factor Va by activated protein C and Factor Xa, as indicated by the data presented in this report, is illustrated schematically in Fig. 5. Fig. 5 also indicates some of the heterogeneity found in Factor Va.

Although the light chain (component E) of bovine Factor Va is most often reported and referred to as a single species by this laboratory and others, it does in fact appear frequently on SDS gels as a doublet (*e.g.*, this paper (Fig. 1), Nesheim *et al.* (9) (Fig. 7), and Guinto and Esmon (10) (Figs. 3 and 4)). Suzuki *et al.* (11) did report a doublet for the light chain of human Factor Va. The difference between the two members of the doublet is most likely near the carboxyl terminus of the molecules since, as discussed below, upon cleavage of component E by either activated protein C or Factor Xa, the amino terminus-derived fragment of M_r = 30,000 appears as a single species, whereas the carboxyl terminus-derived fragments appear as a doublet with each member having the same amino-terminal sequence (Fig. 5). The appearance of component E as a doublet could be due to an as yet undiscovered proteolytic event at the carboxyl terminus of Factor V or to the synthesis of two different species of Factor V.

Additional polypeptide species contribute to the apparent heterogeneity of preparations of component E (Fig. 1, *lane 5*). These molecules appear as bands on SDS gels which migrate in exactly the same positions as the products of activated protein C or Factor Xa proteolysis of component E (compare

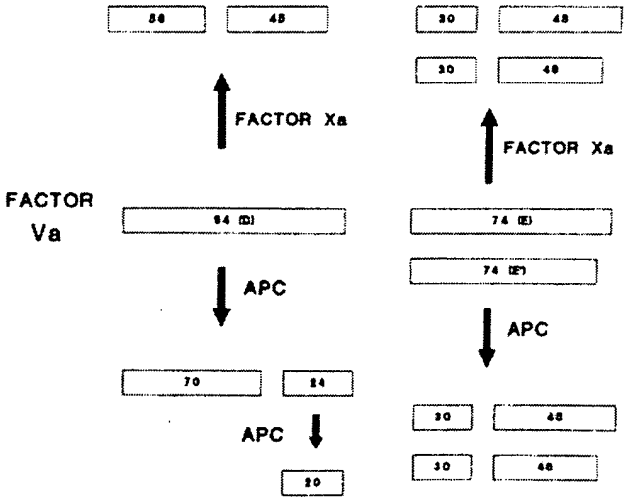

FIG. 5. **Schematic representation of cleavages of components of Factor Va catalyzed by activated protein C and Factor Xa.**

lane 5 with *lane 8* or *9* of Fig. 1). In fact, they probably are the same molecules. Sequence analyses of products of activated protein C or Factor Xa proteolysis of component E show no additional sequence which could be due to a different molecule. The presence of these fragments in preparations of component E is probably due to a combination of the lability of the particular peptide bond to proteolysis and strong noncovalent association between the fragments. The lability of the bond to proteolysis is suggested by the discovery that it is susceptible to cleavage catalyzed by two proteases (activated protein C and Factor Xa) which demonstrate clearly different specificities. The strong noncovalent association between fragments is suggested by the discovery (discussed below) that Factor Va retains complete activity even after component E has been fragmented by activated protein C or Factor Xa cleavage.

The processing of component E by activated protein C and Factor Xa to fragments which migrate identically on SDS gels has been reported previously from this laboratory (8). What we have now demonstrated is that the product sets of the two cleavages appear the same because they are the same: activated protein C and Factor Xa cleave component E at the same position. Additionally, the identity of the amino-terminal sequence of the fragments of $M_r = 30{,}000$ with the amino-terminal sequence of component E indicates that this smaller fragment is derived from the amino-terminal portion of the parent molecule, whereas the larger fragments (doublet) are derived from the carboxyl-terminal portion. The placement of these fragments is illustrated in Fig. 5.

Proteolysis of the heavy chain of Factor Va has been the focus of a great deal of attention (7, 8, 10) because of interest in the activated protein C proteolytic inactivation of Factor Va, whereas the processing of the heavy chain by Factor Xa has received less attention (8). It is clear from the sizes of the fragments that activated protein C and Factor Xa cleave at different positions. Sequencing has allowed us to determine where in the molecule these cleavages occur. In both cases, the larger fragment is derived from the amino-terminal portion of the parent molecule. The placement of these fragments is illustrated in Fig. 5. Activated protein C also cleaves at a second location, converting the fragment of $M_r = 24{,}000$ to one of $M_r = 20{,}000$. The position of this cleavage is similar to that observed earlier (8) to be due to a platelet-associated protease.

The activity measurements using proteolytically processed Factor Va (Fig. 4) confirm that it is an activated protein C cleavage of the heavy chain of Factor Va that causes inactivation of the cofactor (6, 8,10). The activity of Factor Va in the DAPA assay was unchanged over a period of 4 h of incubation with Factor Xa, even though during this time both components D and E were completely cleaved to give rise to the fragments described. In contrast, activated protein C cleavage of Factor Va caused a substantial loss of cofactor activity. Since both activated protein C and Factor Xa cleave at the same position in the light chain and since Factor Xa cleavage does not inactivate Factor Va, then the activated protein C cleavage of the heavy chain must be responsible for inactivation.

It should be noted, however, that even when no heavy chain remains after activated protein C cleavage of Factor Va (Fig. 4, *lanes 5* and *8*), there is residual (30–35%) cofactor activity. Such residual Factor Va activity in the absence of intact heavy chain after activated protein C cleavage has been reported (10), although other reports (5, 7) indicate a greater correlation between activity and amount of intact heavy chain. Our results clearly show residual activity, indicating that the inactivation of Factor Va is a more complex event than simply the cleavage of a single bond.

The sequence homology found between the fragments of bovine Factor V and human Factor VIII is not surprising considering the homology already reported between bovine Factor V and porcine Factor VIII (15, 19) and between porcine and human Factors VIII (16). While not surprising, it is notable that the homology occurs in clusters (*e.g.* FHAING) and that the homology extends to human cerruloplasmin as has been reported for other portions of Factor V (17, 19).

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